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(21) International Application Number: PCT/US98/21258 (22) International Filing Date: 6 October 1998 (06.10.98) (30) Priority Data: 60/061,182 6 October 1997 (06.10.97) US (71) Applicant (for all designated States except US): BASF AK-TIENGESSELLSCHAFT [DE/DE]; D-67056 Ludwigshafen (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): ARNOLD, Lee, D. [CA/US]; 216 Ruggles Street, Westborough, MA 01581 (US). XU, Yajun [CN/US]; 26 Smith Street, Westborough, MA 01581 (US). BARLOZZARI, Teresa [IT/US]; 24 South Woodside Avenue, Wellesley, MA 02181 (US). (74) Agents: WAGNER, Richard, W. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: INDENO[1,2-c]-, NAPHTHO[1,2-c]- AND BENZO[6,7]CYCLOHEPTA[1,2-c]PYRAZOLE DERIVATIVES (57) Abstract <p>Chemical compounds that are derivatives of indeno[1,2-c]-, naphtho[1,2-c]- and benzo[6,7]cyclohepta[1,2-c]pyrazole are inhibitors of tyrosine kinase activity. Several of the tyrosine kinases, whose activity is inhibited by these chemical compounds, are involved in hyperproliferative, angiogenic or in vascular hyperpermeability processes. Thus, these chemical compounds can ameliorate disease states where tumor formation, metastasis, angiogenesis, vascular hyperpermeability or endothelial cell proliferation is a factor.</p>		

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**INDENO[1,2-c]-, NAPHTHO[1,2-c]- AND
BENZO[6,7]CYCLOHEPTA[1,2-c]PYRAZOLE DERIVATIVES**

5 BACKGROUND OF THE INVENTION

Protein tyrosine kinases (PTKs) comprise a large and diverse class of proteins having enzymatic activity. The PTKs play an important role in the control of cell growth and differentiation (for review, *see* Schlessinger & Ullrich, 1992, *Neuron* 9:383-391).

10 For example, receptor tyrosine kinase mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), typically followed by receptor dimerization, transient stimulation of the intrinsic protein tyrosine kinase activity and phosphorylation. Binding sites are thereby created for intracellular signal transduction molecules and lead to the formation of complexes
15 with a spectrum of cytoplasmic signalling molecules that facilitate the appropriate cellular response. (*e.g.*, cell division, metabolic effects, responses to the extracellular microenvironment) *see* Schlessinger and Ullrich, 1992, *Neuron* 9:1-20.

With respect to receptor tyrosine kinases, it has been shown also that tyrosine phosphorylation sites function as high-affinity binding sites for SH2 (*src* homology)
20 domains of signaling molecules (Fantl *et al.*, 1992, *Cell* 69:413-423; Songyang *et al.*, 1994, *Mol. Cell. Biol.* 14:2777-2785; Songyang *et al.*, 1993, *Cell* 72:767-778; and Koch *et al.*, 1991, *Science* 252:668-678). Several intracellular substrate proteins that associate with receptor tyrosine kinases (RTKs) have been identified. They may be divided into two principal groups: (1) substrates which have a catalytic domain;
25 and (2) substrates which lack such a domain but serve as adapters and associate with catalytically active molecules (Songyang *et al.*, 1993, *Cell* 72:767-778). The specificity of the interactions between receptors or proteins and SH2 domains of their substrates is determined by the amino acid residues immediately surrounding the phosphorylated tyrosine residue. Differences in the binding affinities between
30 SH2 domains and the amino acid sequences surrounding the phosphotyrosine residues on particular receptors are consistent with the observed differences in their substrate phosphorylation profiles (Songyang *et al.*, 1993, *Cell* 72:767-778). These

observations suggest that the function of each receptor tyrosine kinase is determined not only by its pattern of expression and ligand availability but also by the array of downstream signal transduction pathways that are activated by a particular receptor. Thus, phosphorylation provides an important regulatory step which determines the selectivity of signalling pathways recruited by specific growth factor receptors, as well as differentiation factor receptors.

Aberrant stimulation, expression or mutations in the PTKs have been shown to lead to either uncontrolled cell proliferation (e.g., malignant tumor growth) or to defects in key developmental or reparative processes. Consequently, the biomedical community has expended significant resources to discover the specific biological role of members of the PTK family, their function in differentiation processes, their involvement in tumorigenesis and in other diseases, the biochemical mechanisms underlying their signal transduction pathways activated upon ligand stimulation and the development of novel drugs.

Tyrosine kinases can be of the receptor-type (having extracellular, transmembrane and intracellular domains) or the non-receptor type (being wholly intracellular).

Receptor Tyrosine Kinases (RTKs). The RTKs comprise a large family of transmembrane receptors with diverse biological activities. The receptor tyrosine kinase (RTK) family includes receptors that are crucial for the growth and differentiation of a variety of cell types (Yarden and Ullrich, *Ann. Rev. Biochem.* 57:433-478, 1988; Ullrich and Schlessinger, *Cell* 61:243-254, 1990). The intrinsic function of RTKs is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses (Ullrich & Schlessinger, 1990, *Cell* 61:203-212).

At present, at least nineteen (19) distinct RTK subfamilies have been identified. One RTK subfamily, designated the HER subfamily, is believed to be comprised of EGFR, HER2, HER3, and HER4. Ligands to the HER subfamily of receptors include epithelial growth factor (EGF), TGF- α , amphiregulin, HB-EGF, betacellulin and heregulin. Aberrant regulation of HER2/erbB2 kinase activity is believed to promote a transformed tumorigenic phenotype especially in breast

carcinomas. Two other RTK subfamilies are designated the insulin receptor subfamily, which is comprised of INS-R, IGF-1R and IR-R, and "PDGFR" subfamily which includes the PDGF α and β -receptors, CSF-1R, and c-kit.

Several receptor tyrosine kinases, and growth factors that bind thereto, have
5 been suggested to play a role in angiogenesis, although some may promote angiogenesis indirectly (Mustonen and Alitalo, *J. Cell Biol.* 129:895-898, 1995). One such receptor tyrosine kinase, known as fetal liver kinase 1 (flk-1), is a member of the type III subclass of RTKs. An alternative designation for human flk-1 is kinase insert domain-containing receptor (KDR) (Terman *et al.*, *Oncogene* 6:1677-
10 83, 1991). Another alternative designation for flk-1/KDR is "vascular endothelial cell growth factor receptor 2" (VEGFR-2). The murine version of flk-1/VEGFR-2 has also been called NYK (Oelrichs *et al.*, *Oncogene* 8 (1):11-15, 1993). DNAs encoding mouse, rat and human flk-1 have been isolated, and the nucleotide and encoded amino acid sequences reported (Matthews *et al.*, *Proc. Natl. Acad. Sci.*
15 *USA*, 88:9026-30, 1991; Terman *et al.*, 1991, *supra*; Terman *et al.*, *Biochem. Biophys. Res. Comm.* 187:1579-86, 1992; Sarzani *et al.*, *supra*; and Millauer *et al.*, *Cell* 72:835-846, 1993).

The type III subclass RTK designated *fms*-like tyrosine kinase-1 (flt-1) is related to flk-1/KDR (DeVries *et al.* *Science* 255:989-991, 1992; Shibuya *et al.*,
20 *Oncogene* 5:519-524, 1990). An alternative designation for flt-1 is "vascular endothelial cell growth factor receptor 1" (VEGFR-1). To date, members of the flk-1/KDR/VEGFR-2 and flt-1/ VEGFR-1 subfamilies have been found expressed primarily on endothelial cells. These subclass members are specifically stimulated by members of the vascular endothelial cell growth factor (VEGF) family of ligands
25 (Klagsburn and D'Amore, *Cytokine & Growth Factor Reviews* 7: 259-270, 1996). Flt-1 is believed to be essential for endothelial organization during vascular development. Flt-1 expression is associated with early vascular development in mouse embryos, and with neovascularization during wound healing (Mustonen and Alitalo, *supra*). Expression of flt-1 in adult organs suggests an additional function
30 for this receptor that is not related to cell growth (Mustonen and Alitalo, *supra*).

Another RTK that is related to flt-1 and flk-1/KDR is flt-4 (Galland *et al.*, *Oncogene* 8:1233-40, 1993; Pajusola *et al.*, *Oncogene* 8:2931-37, 1993). Features shared by these three receptors include the seven immunoglobulin-like domains in their extracellular region. The amino acid sequence of flt-4 exhibits significant
5 homology with the sequences of flt-1 and flk-1/KDR, especially in the tyrosine kinase domain (Galland *et al.*, *supra*). Unlike flt-1 and flk-1, however, a precursor form of flt-4 is cleaved during post-translational processing to form two disulfide-linked polypeptides (Pajusola *et al.*, *supra*). Studies of flt-4 expression during development support the theory of venous origin of lymphatic vessels (Kaipainen *et al.*, *Proc. Natl. Acad. Sci. USA* 92:3566-70, April, 1995).
10

Given the crucial role of endothelial cells in angiogenesis, growth factors that act on endothelial cells are of particular interest for studies of the regulation of vascularization. One such factor is vascular endothelial cell growth factor (VEGF), which binds to both flk-1/KDR and flt-1 with relatively high affinity and is
15 mitogenic toward vascular endothelial cells (Terman *et al.*, 1992, *supra*; Mustonen *et al.* *supra*; DeVries *et al.*, *supra*). VEGF does not bind to flt-4 (Pajusola *et al.*, *supra*). The studies reported in Millauer *et al.*, *supra*, suggest that VEGF and flk-1/KDR/VEGFR-2 are a ligand-receptor pair that play an important role in the formation and sprouting of blood vessels, termed vasculogenesis and angiogenesis,
20 respectively.

Different forms of VEGF arising from alternative splicing of mRNA have been reported, including the four species described by Ferrara *et al.* (*J. Cell. Biochem.* 47:211-218, 1991). Both secreted and predominantly cell-associated species of VEGF were identified by Ferrara *et al.* *supra*, and the protein is known to
25 exist in the form of disulfide linked dimers.

It is also known that there are a variety of physiological and biochemical mechanisms that underlie edema and the formation of the edematous state in an individual. An important mediator in one or more of these mechanisms is "vascular endothelial cell growth factor" (VEGF). This mediator is also known as "vascular
30 permeability factor" (VPF). This factor upregulates transport in vascular endothelial cells, and causes an increase in the permeability of numerous vascular beds

including the skin, subcutaneous tissues, peritoneal wall, mesentery, diaphragm, trachea, bronchi, duodenum and uterus. Significant diapedesis, alterations in exchange across the endothelium, extravasation and deposition of macromolecules at these sites and prolonged hypotension may accompany these increased permeability effects. These processes are thought to be a facilitating prelude to neovascularization. VEGF is expressed by inflammatory T-cells, macrophages, neutrophils and eosinophils, etc. at sites of inflammation. This factor is upregulated by hypoxia, certain vasopressor hormones, growth factors, reproductive hormones and numerous inflammatory cytokines. VEGF-mediated vascular permeability has been implicated in such disorders as tumor ascites, endometriosis, edematous responses to burns and trauma, endothelial dysfunction in diabetes, ovarian hyperstimulation syndrome complications, and ocular edema.

Thus, it is apparent that the inhibition of VEGF production or activity would be beneficial, especially to block the manifestation of the above-listed disorders. In particular, agents that are capable of blocking VEGF mediated hyperpermeability and edema and associated syndromes would be useful for alleviating these disorders.

Placenta growth factor (PlGF) has an amino acid sequence that exhibits significant homology to the VEGF sequence (Park *et al.*, *J. Biol. Chem.* 269:25646-54, 1994; Maglione *et al. Oncogene* 8:925-31, 1993). As with VEGF, different species of PlGF arise from alternative splicing of mRNA, and the protein exists in dimeric form (Park *et al.*, *supra*). PlGF binds flt-1 with high affinity, but not flk-1/KDR (Park *et al.*, *supra*). PlGF potentiates the mitogenic effect of VEGF on endothelial cells when VEGF is present at low concentrations, but has no detectable effect when VEGF is present at higher concentrations (Park *et al.*, *supra*).

Two other subfamilies of RTKs have been designated as the FGF receptor family (FGFR1, FGFR2, FGFR3 and FGFR4) and the Met subfamily (c-met and Ron).

Because of the similarities between the PDGF and FLK subfamilies, the two subfamilies are often considered together. The known RTK subfamilies are identified in Plowman *et al.*, 1994, *DN&P Z(6)*:334-339, which is incorporated herein by reference.

Inhibiting angiogenesis is desirable in certain clinical situations (e.g., to suppress growth and metastasis of solid tumors, or in treating rheumatoid arthritis), whereas promotion of vascularization is beneficial for treating other conditions (e.g., wound healing). Consequently, molecules that promote angiogenesis by transducing
5 signals through the above-discussed receptors, and molecules capable of inhibiting such signal transduction, are both of interest.

The Non-Receptor Tyrosine Kinases. The non-receptor tyrosine kinases represent a collection of cellular enzymes which lack extracellular and transmembrane sequences. At present, over twenty-four individual non-receptor
10 tyrosine kinases, comprising eleven (11) subfamilies (Src, Frk, Btk, Csk, Abl, Zap70, Fes/Fps, Fak, Jak, Ack and LIMK) have been identified. At present, the Src subfamily of non-receptor tyrosine kinases is comprised of the largest number of PTKs and include Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and Yrk. The Src subfamily of enzymes has been linked to oncogenesis. A more detailed discussion
15 of non-receptor tyrosine kinases is provided in Bolen, 1993, *Oncogene* 8:2025-2031, which is incorporated herein by reference.

Many of the tyrosine kinases, whether an RTK or non-receptor tyrosine kinase, have been found to be involved in cellular signaling pathways involved in numerous pathogenic conditions, including cancer, psoriasis, and other
20 hyperproliferative disorders or hyper-immune responses.

Development of Compounds to Modulate the PTKs. In view of the surmised importance of PTKs to the control, regulation, and modulation of cell proliferation, the diseases and disorders associated with abnormal cell proliferation, many attempts have been made to identify receptor and non-receptor tyrosine kinase
25 "inhibitors" using a variety of approaches, including the use of mutant ligands (U.S. Application No. 4,966,849), soluble receptors and antibodies (Application No. WO 94/10202; Kendall & Thomas, 1994, *Proc. Natl. Acad. Sci* 90:10705-09; Kim *et al.*, 1993, *Nature* 362:841-844), RNA ligands (Jellinek, *et al.*, *Biochemistry* 33:10450-56; Takano, *et al.*, 1993, *Mol. Bio. Cell* 4:358A; Kinsella, *et al.* 1992, *Exp. Cell Res.*
30 199:56-62; Wright, *et al.*, 1992, *J. Cellular Phys.* 152:448-57) and tyrosine kinase

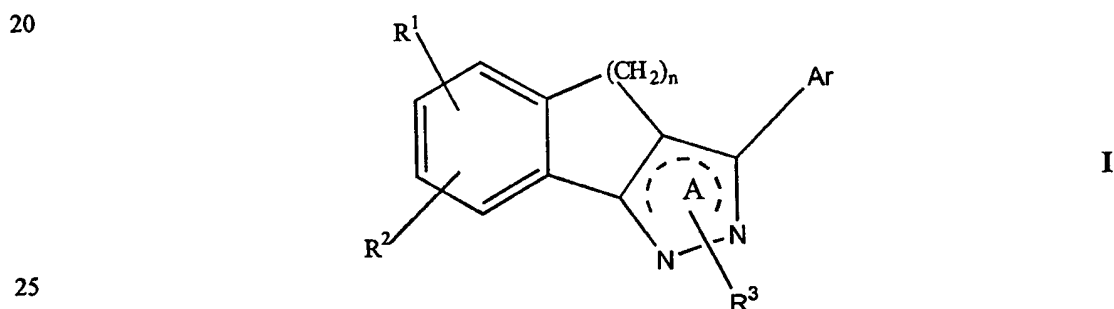
inhibitors (WO 94/03427; WO 92/21660; WO 91/15495; WO 94/14808; U.S. Patent No. 5,330,992; Mariani, *et al.*, 1994, *Proc. Am. Assoc. Cancer Res.* 35:2268).

More recently, attempts have been made to identify small molecules which act as tyrosine kinase inhibitors. For example, bis monocyclic, bicyclic or heterocyclic aryl compounds (PCT WO 92/20642), and vinylene-azaindole derivatives (PCT WO 94/14808) have been described generally as tyrosine kinase inhibitors. Styryl compounds (U.S. Patent No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Patent No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266 A1), seleoindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic compounds (PCT WO 92/21660) and benzylphosphonic acid compounds (PCT WO 91/15495) have been described as compounds for use as tyrosine kinase inhibitors for use in the treatment of cancer.

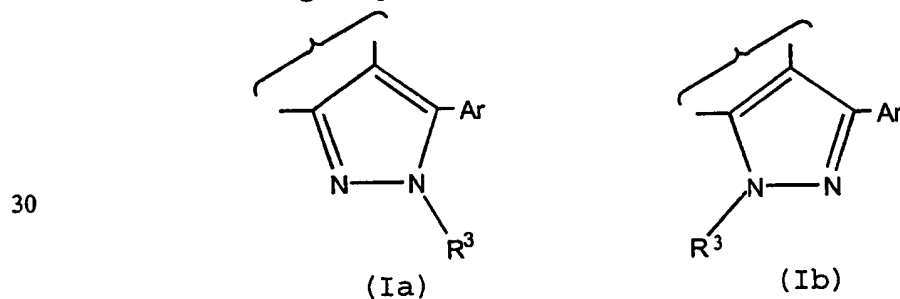
The identification of effective small compounds which specifically inhibit tyrosine signal transduction by modulating the activity of receptor and non-receptor tyrosine kinases to regulate and modulate abnormal or inappropriate cell proliferation is therefore desirable.

SUMMARY OF THE INVENTION

This invention is directed to compounds of the formula:

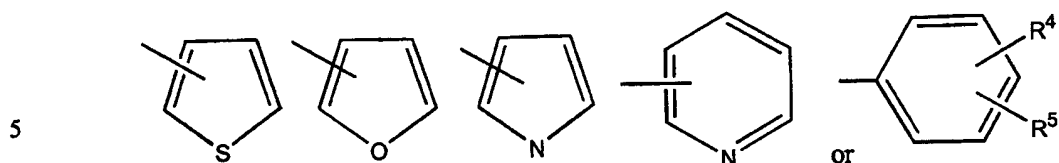


wherein ring A represents the structures:



n is 1, 2, or 3;

Ar is



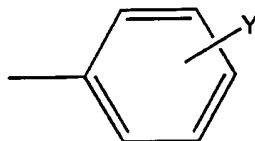
and

10 R¹, R² and R⁴ and R⁵ each independently represent hydrogen, halogen having an atomic weight of about 19 to 36, lower alkyl having 1 to 4 carbon atoms, lower alkoxy, trifluoromethyl or

R¹, R² and R⁴, R⁵ together independently represent methylenedioxy attached to adjacent carbon atoms;

15 R³ is hydrogen, COR⁶, or SO₂R⁶

R⁶ is -CH₃ or



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wherein Y is hydrogen, chlorine or -CH₃.

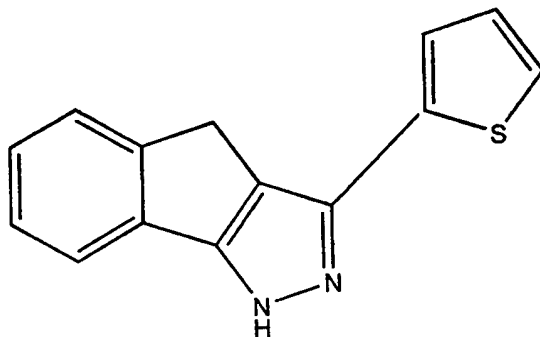
Enantiomers, tautomers, and mixtures of these compounds are included in this invention. Pharmaceutically acceptable acid addition salts of these compounds are also included in this invention.

25 The compounds of this invention are useful as inhibitors of the tyrosine kinase activity. In particular, the compounds of this invention are useful as inhibitors of tyrosine kinases that are important in the process of angiogenesis. These compounds are also useful as inhibitors of tyrosine kinases that are important in the process of vascular hyperpermeability. Since these compounds are anti-
30 angiogenic and inhibit vascular hyperpermeability, they are important substances for

inhibiting the progression of disease states where angiogenesis and vascular hyperpermeability are important components.

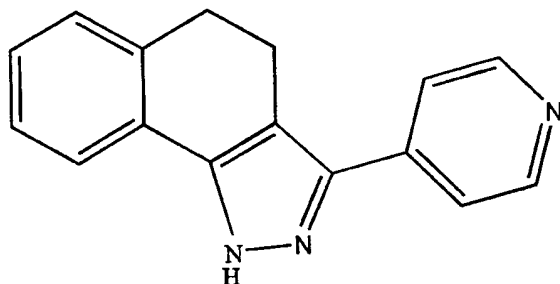
Preferred compounds of the present invention have the formulae:

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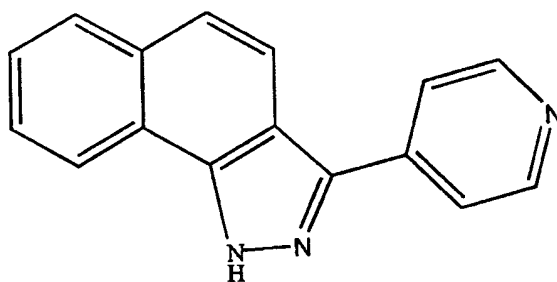
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and

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The present invention further includes the use of these compounds in pharmaceutical compositions with a pharmaceutically effective amount of the above-described compounds and a pharmaceutically acceptable carrier or excipient. These pharmaceutical compositions can be administered to individuals to slow or halt the process of angiogenesis in angiogenesis-aided diseases or of vascular hyperpermeability in diseases associated with this process.

DETAILED DESCRIPTION OF THE INVENTION

The compounds of this invention have antiangiogenic properties. For this reason, these compounds can be used as active agents against such disease states as
5 arthritis, atherosclerosis, psoriasis, hemangiomas, myocardial angiogenesis, coronary and cerebral collaterals, ischemic limb angiogenesis, wound healing, peptic ulcer *Helicobacter* related diseases, fractures, cat scratch fever, rubeosis, neovascular glaucoma and retinopathies such as those associated with diabetic retinopathy or age-related macular degeneration. In addition, some of these compounds can be
10 used as active agents against solid tumors and hyperproliferative disorders since such diseases require a proliferation of blood vessel cells for growth and metastasis.

The compounds of this invention have inhibitory activity against tyrosine kinases. That is, these compounds modulate signal transduction by tyrosine kinases. In particular, these compounds selectively inhibit the activity of the KDR/FLK-
15 1/VEGFR-2 tyrosine kinases. Certain compounds of this invention also inhibit the activity of additional tyrosine kinases such as Flt-1/VEGFR-1, Src-subfamily kinases such as Lck, Src, fyn, yes, etc. The occurrence and potency of the inhibitory activity of the compounds of this invention against a particular tyrosine kinase is dependent on the nature, number and arrangement of the substituents (i.e., R¹, R², R³,
20 R⁴, R⁵ and R⁶) of these compounds.

The compounds of this invention, when administered to individuals in need of such compounds, also inhibit vascular hyperpermeability and the formation of edema in these individuals. These compounds act, it is believed, by inhibiting the activity mediated by VEGF receptors which are involved in the process of vascular
25 hyperpermeability and edema formation. VEGF is unique in that it is the only angiogenic growth factor known to contribute directly to vascular hyperpermeability and the formation of edema. Indeed, vascular hyperpermeability and edema that is associated with the expression or administration of many other growth factors appears to be mediated via VEGF production. Diapedesis also often accompanies
30 vascular hyperpermeability. Similarly, excessive vascular hyperpermeability can disrupt normal molecular exchange across the endothelium in critical tissues and

organs (e.g., lung and kidney), thereby perturbing function and causing macromolecular extravasation and deposition. By inhibiting the kinase activity of VEGF receptors, hyperpermeability, as well as associated extravasation, subsequent edema or ascites formation and matrix deposition is inhibited and minimized.

5 In addition, the N-acylated ($R^3 = -COR^6$) forms or N-sulfonylated ($R^3 = -SO_2R^6$) analogs of the compounds of this invention can also serve as prodrugs which act as metabolically activated antiangiogenic agents.

It is envisaged that the disorders listed above are mediated to a significant extent by protein tyrosine kinase activity involving the KDR/VEGFR-2 and/or the
10 Flt-1/VEGFR-1 tyrosine kinases. By inhibiting the activity of these tyrosine kinases, the progression of the listed disorders is inhibited because the angiogenic component of the disease state is severely curtailed. The action of the compounds of this invention, by their selectivity for specific tyrosine kinases, result in a minimization of side effects that would occur if less selective tyrosine kinase
15 inhibitors were used.

The potency and specificity of the generic compounds of this invention can often be altered and optimized by substituent variations and conformational restrictions.

In this invention, the following definitions are applicable:

20 "Pharmaceutically acceptable acid addition salt" refers to those salts which retain the biological effectiveness and properties of the free bases and which are obtained by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid or organic acids such as sulfonic acid, carboxylic acid, organic phosphoric acid, methanesulfonic acid, ethanesulfonic acid,
25 p-toluenesulfonic acid, salicylic acid, lactic acid, tartaric acid and the like.

"Alkyl" refers to a saturated aliphatic hydrocarbon, including straight-chain and branched-chain groups having 1 to 4 carbons.

"Alkoxy" refers to an "O-alkyl" group, where "alkyl" is defined as described above.

Pharmaceutical Formulations and Routes of Administration

The identified compounds of this invention can be administered to a human patient by themselves or in pharmaceutical compositions where they are mixed with suitable carriers or excipient(s) at doses to treat or ameliorate a variety of disorders.

- 5 A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

10

Routes of Administration

- Suitable routes of administration may, for example, include oral, eyedrop, rectal, transmucosal, topical, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as
15 intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the compound in a local rather than a systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

- 20 Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

Composition/Formulation

- 25 The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

- Pharmaceutical compositions for use in accordance with the present
30 invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which

facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by combining the active compound with a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a

plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g. bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran.

Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution
5 with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may
10 also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly or by intramuscular injection). Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for
15 example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the
20 nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be
25 varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or
30 polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the PTK modulating compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protic solvents than are the corresponding free base forms.

Effective Dosage

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cellular assays. For example, a dose

can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cellular assays (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the PTK activity). Such information can be used to more accurately determine useful doses in humans.

- 5 A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the
- 10 dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50} . Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such
- 15 compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl *et al.*, 1975, in "The
- 20 Pharmacological Basis of Therapeutics", Ch. 1 p1).

- Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data; e.g. the concentration necessary
- 25 to achieve 50-90% inhibition of the kinase using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

- Dosage intervals can also be determined using the MEC value. Compounds
- 30 should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably

between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient.

10 The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition. Suitable conditions indicated on the

15 label may include treatment of a cellular hyperproliferative disorder, inhibition of angiogenesis, treatment of fibrosis, diabetes, edema, ascites and the like.

EXEMPLIFICATIONS

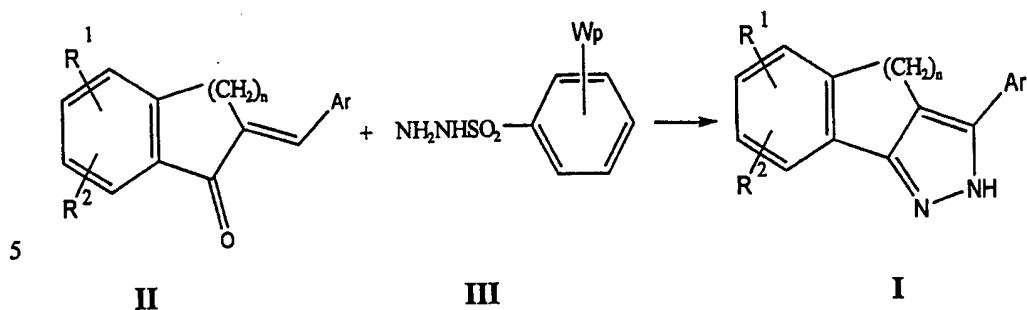
I. Synthesis

20 There are two general approaches to the synthesis of the indeno-, naphtho-, and cyclohepta[1,2-c]pyrazole ring systems that have been set forth in U.S. Patent 3,843,665 and U.S. Patent 3,843,666 and represented by formula I.

In U.S. Patent 3,843,665, cyclization of the pyrazole ring is affected by heating compounds of the structure II with an aromatic sulfonylhydrazide in an inert

25 solvent and a catalytic amount of an acid. The reaction is carried out for a period of 5 to 30 hours at a temperature of 75 to 100 C. The following scheme is representative of this transformation.

-19-

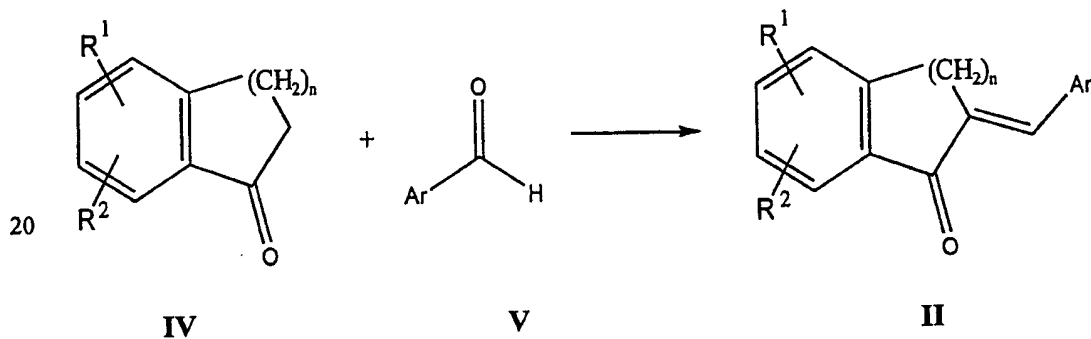


Wherein:

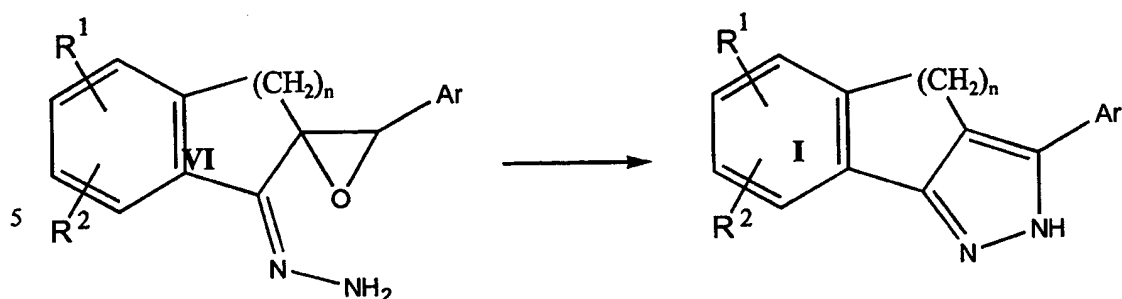
- 10
- | | |
|---|-------------------|
| p | is 0, 1, 2; and |
| W | is a lower alkyl. |

Compounds of formula **II** are prepared by treating an appropriately functionalized 1-indanone, alpha-tetralone, or 1-benzosuberone with an arylaldehyde in the presence of an acid or base catalyst (Braun, R. A.; Mosher, W. A. *J. Amer.*

15 *Chem. Soc.* 1958, 80, 2749).



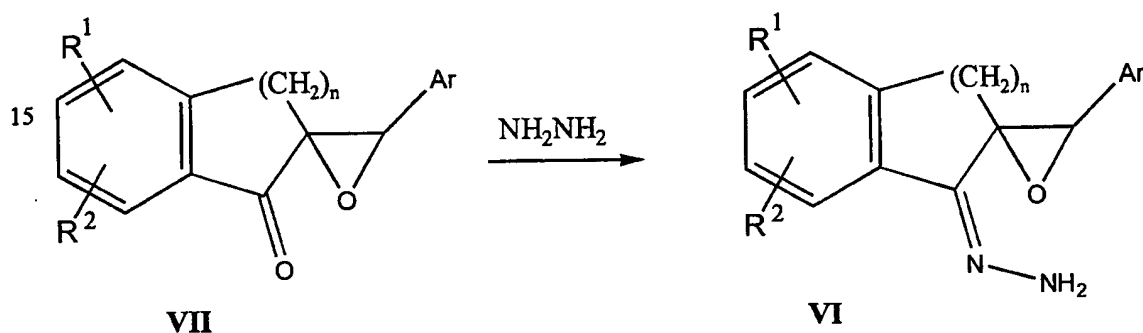
25 A second method of preparing indeno-, naphtho-, and benzo[6,7]cyclohepta[1,2-c]pyrazole is set forth by U.S. Patent 3,843,666 where compounds with the general formula **VI** are heated to 75° to 175°C with a catalytic amount of an organic carboxylic acid or an organic sulfonic acid in an inert solvent such as an aromatic hydrocarbon for a period of 6 to 24 hours.



Wherein:

Ar, R¹ and R² are as set forth above.

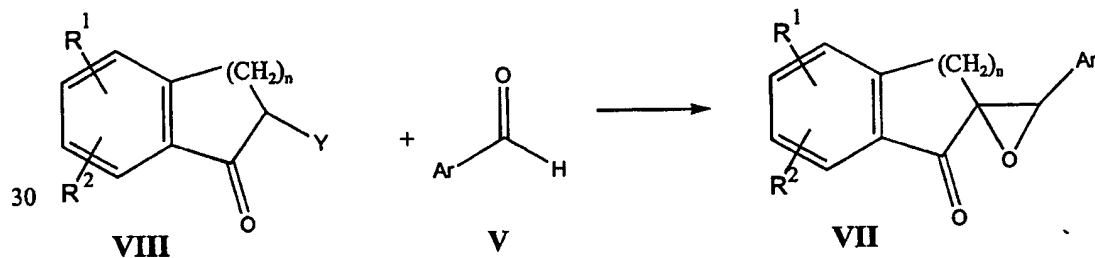
10 Compounds which have the general formula of VI are prepared by treating compounds of the general formula VII with hydrazine in an inert solvent. The reaction is carried out at 15° to 20°C for a period of up to 24 hours.



20

Compounds which have the general formula VII are prepared by treating II with alkaline hydrogen peroxide at ambient temperature in a suitable organic cosolvent mixture such as CH₂Cl₂/MeOH. Alternatively, VII is produced from the reaction of VIII with an aryl aldehyde under basic conditions. The reaction is

25 carried out in an inert solvent at a temperature between 5° and 10°C for a period of 3 to 6 hours.

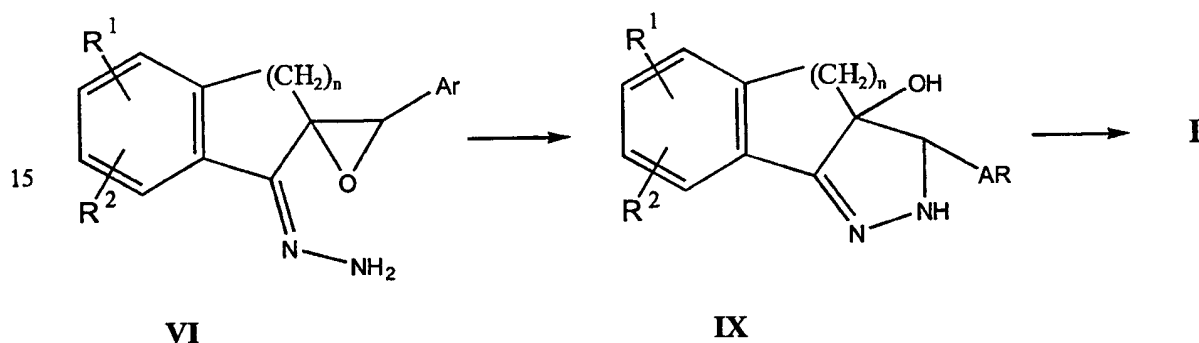


Wherein:

Y is any conventional leaving group, such as chlorine, bromine, iodine, tosylate or mesylate.

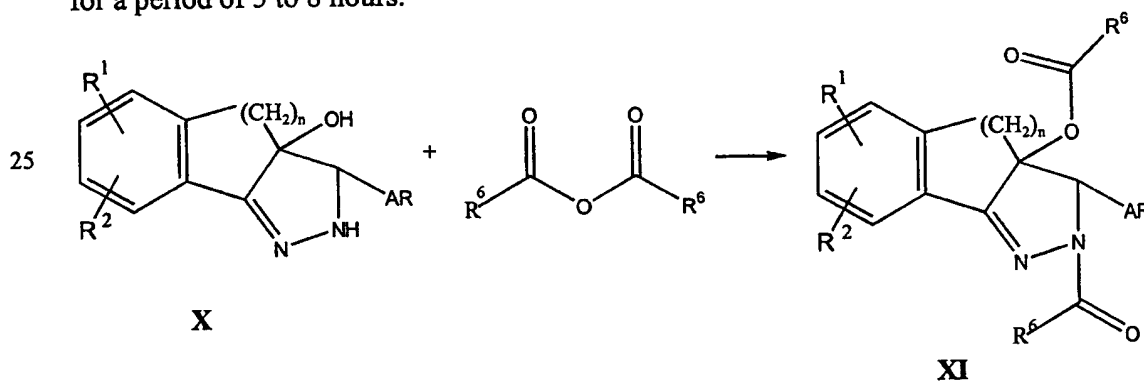
5

Cyclization of VI can also be affected by treatment with a mineral acid such as hydrochloric acid, sulfuric acid or phosphoric acid. The reaction is carried out in a lower alkanol at a temperature between 15° to 20°C for a period of 12 to 48 hours. The product of the reaction, IX, can then be aromatized to I by heating to a
 10 temperature of 50° to 150°C with an organic carboxylic acid or an organic sulfonic acid in a straight chain ether or cyclic ether for the period of 8 to 30 hours.



20

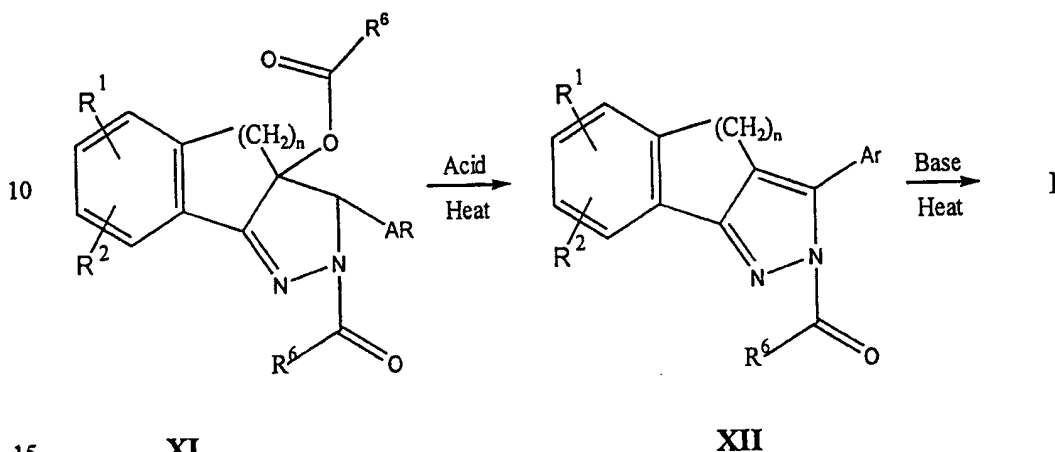
Compound IX can be diacetylated by treatment with acetic anhydride in an inert solvent such as an aromatic hydrocarbon at a temperature between 35° to 200°C for a period of 5 to 8 hours.



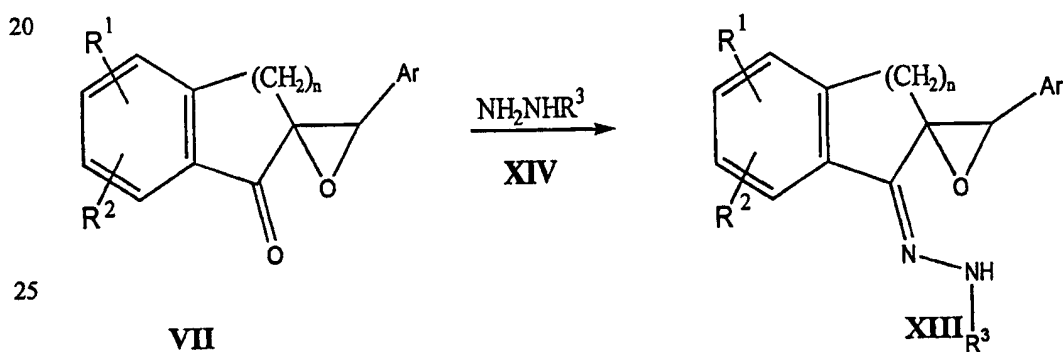
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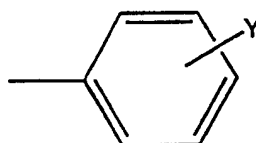
Compound **XI** can then be aromatized to compound **XII** by heating to a temperature of 35° to 200°C with a mineral acid or an organic acid in an inert solvent for the period of 4 to 8 hours. Finally, compound **XII** can be converted to **I** by heating to a temperature of 50° to 150°C in an inert solvent such as water or a lower alcohol in the presence of an alkali metal or an alkali metal hydroxide for the period of 8 to 30 hours.



Compounds with the general formula **XIII** can be obtained by treating **VII** with a compound of the formula **XIV** in the presence of an inert solvent such as a lower alkanol, straight chain ether or cyclic ether at a temperature between 15° to 20°C for a period of 24 hours.

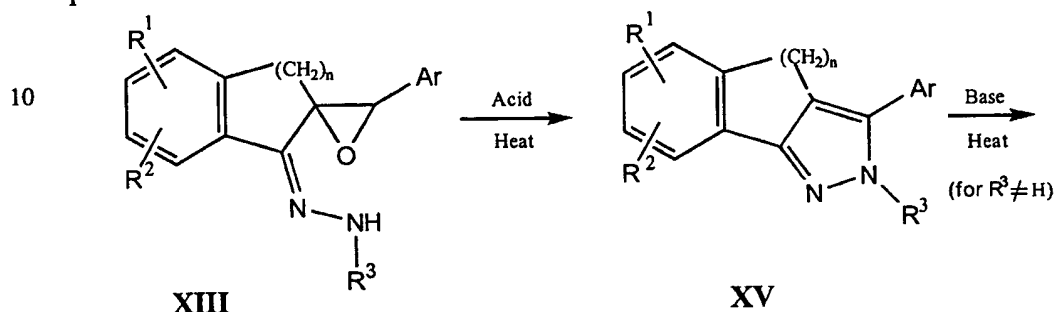
**VII**

Wherein:

 R^3 is hydrogen, $-COR^6$, or $-SOR^6$ and R^6 is $-CH_3$ or

Y is hydrogen, chlorine or $-\text{CH}_3$.

Compounds with the general formula **XIII** can be cyclized to **XV** by heating to 75° to 175°C with a catalytic amount of an organic carboxylic acid or an organic sulfonic acid in an inert solvent such as an aromatic hydrocarbon for the period of 6 to 24 hours. Compounds with the general formula **XV** ($\text{R}^3 \neq \text{H}$) can be converted to **I** by heating to a temperature of 50° to 150°C in an inert solvent such as water or a lower alcohol in the presence of an alkali metal or an alkali metal hydroxide for the period of 8 to 30 hours.



Specific examples of the above transformations can be found in U.S. Patents 3,843,665 and 3,843,666.

Examples

The invention is further illustrated by the following Examples which are given by way of example only. The final product of each of these Examples was characterized by one or more of the following procedures: high performance liquid chromatography; elemental analysis, nuclear magnetic resonance spectroscopy, infrared spectroscopy and high resolution mass spectroscopy:

- THF = tetrahydrofuran;
- DMF = dimethylformamide;
- IMS = industrial methylated spirits; and
- LCMS = liquid chromatography/mass spectroscopy.

Examples 1-3 were prepared by methods given in US 3,932,430 (Sandoz, 1976):

Example 1

3-(3-pyridyl)-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 223-224°C;

Example 2

5 3-(4-pyridyl)-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 264-266°C (dec);

Example 3

3-(2-thienyl)-4,5-dihydro-1*H*-benz[g]indazole, m.p. 206-208°C.

10 Example 4

A mixture of 2-benzylidene-indan-1-one (3.99 g), p-toluenesulphonyl hydrazine (4.0 g), p-toluenesulphonic acid hydrate (0.7 g) and ethanol (60 ml) was boiled under reflux for 1.5 hours and then left to stand for 18 hours at ambient temperature. The mixture was boiled under reflux for a further 74 hours and then
15 left to stand at ambient temperature for 74 hours. The solvent was removed under reduced pressure and the residue was partitioned between dichloromethane (100 ml) and 2M sodium hydroxide solution (50 ml). The organic layer was separated, dried over magnesium sulfate and filtered. The magnesium sulfate was washed with ethyl acetate and filtered. The ethyl acetate filtrate was evaporated to dryness to give a
20 solid which was recrystallized from ethanol to give 3-phenyl-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 234-236°C.

Example 5

- a) A mixture of indan-1-one (10.0 g), ethanol (35 ml), hydrazine hydrate
25 (10.0 ml) and glacial acetic acid (2.0 ml) was boiled under reflux under nitrogen for 1 hour. The mixture was cooled to 20°C and the mixture concentrated under reduced pressure to give a solid which was collected by filtration to give indan-1-one hydrazone, m.p. 84-86°C.
- 30 b) The hydrazone from a) (3.55 g), was dissolved in tetrahydrofuran (80 ml) and cooled to 0°C under nitrogen while a solution of n-butyl-lithium (28.8 ml of a

2.5M solution in hexane) was added. The mixture was stirred at 0°C for 30 minutes and then ethyl 3-methoxybenzoate (2.16 g) was added and the mixture was stirred at 0°C for 20 minutes. 3M Hydrochloric acid (80 ml) was added and the mixture was boiled under reflux for 1 hour. The organic layer was separated and the aqueous layer was neutralized with sodium bicarbonate and extracted with ether to give an oil which is purified by flash column chromatography using 30-40% ethyl acetate in petroleum ether, b.p. 60-80°C as the mobile phase to give 3-(3-methoxyphenyl)-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 172-174°C.

10 Example 6

A mixture of 2-(4-methoxybenzoyl)indan-1,3-dione (1.4 g, prepared by a modification of the method disclosed in J. Het. Chem. 8, 855-859 (1971)), and hydrazine hydrate (15 ml) was boiled under reflux for 24 hours. The mixture was cooled, poured into ice-water and the solid which precipitated was collected by filtration, washed with absolute ethanol and dried to give 3-(4-methoxyphenyl)-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 247°C.

Example 7

A mixture of 2-(4-methylbenzoyl)indan-1,3-dione (1.32 g, prepared in a similar manner to the starting material in Example 6) and hydrazine hydrate (15 ml) was boiled under reflux for 24 hours. The mixture was cooled, poured into ice-water and the solid was collected by filtration, washed with absolute ethanol and dried to give 3-(p-tolyl)-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 267-269°C.

25 Example 8

a) A solution of 5-methoxyindan-1-one (5.46 g) and *tert*-butyl carbazate (4.45 g) in ethanol (100 ml) and glacial acetic acid (1 drop) was stirred and boiled under reflux for 2 hours. The volume was reduced by half under reduced pressure and the solid which crystallized was collected by filtration to give 5-methoxyindan-1-one *tert*-butyloxycarbonylhydrazone.

- b) A solution of lithium diisopropylamide (11.3 ml of a 2.0M solution in heptane/THF/ethylbenzene) was added dropwise to a stirred solution of 5-methoxyindan-1-one *tert*-butyloxycarbonylhydrazone (2.5 g) in tetrahydrofuran (100 ml) at -78°C under nitrogen. The mixture was stirred at this temperature for 1.5 hours and then a solution of ethyl thiophene-2-carboxylate (1.69 g) in tetrahydrofuran (10 ml) was added dropwise. After the addition the mixture was stirred for 30 minutes at -78°C and then allowed to warm to ambient temperature. After stirring for a further 20 minutes at ambient temperature, saturated ammonium chloride solution (100 ml) was added and the mixture was stirred vigorously for ten minutes. The layers were separated and the aqueous layer was extracted with ether (3 x 75 ml). The combined organics were washed with 1M hydrochloric acid, then brine, and then dried, filtered and evaporated to give a yellow gum which was suspended in dichloromethane (25 ml) and pentamethylbenzene (1.34 g) was added. The mixture was cooled to 0°C and trifluoroacetic acid (0.2 ml) was added. The mixture was stirred for 64 hours and then concentrated under reduced pressure to give an oil which was dissolved in ether (200 ml), washed with saturated sodium bicarbonate solution (100 ml), then brine, then dried, filtered and evaporated to give a gum which was purified by flash column chromatography using petroleum ether, b.p. 60-80°C/ethyl acetate (4:1) as the mobile phase to give 6-methoxy-2-*tert*-butyloxycarbonyl-3-(2-thienyl)-1,4-dihydroindeno[1,2-*c*]pyrazole, m.p. 163-165°C.
- c) 3M Hydrochloric acid (10 ml) was added to a stirred solution of the product of b) (100 mg) in ethyl acetate (10 ml) at ambient temperature. The mixture was stirred at ambient temperature for 1 hour and then heated to reflux. The mixture was boiled under reflux for 30 minutes and then cooled. The mixture was poured into water (50 ml) and the organic layer was separated and washed with water. The combined aqueous layer and washings were made basic with saturated sodium bicarbonate solution and extracted with dichloromethane. The combined dichloromethane washings and extracts were washed with brine, dried, filtered and evaporated to give 6-methoxy-3-(2-thienyl)-1,4-dihydroindeno[1,2-*c*]pyrazole, m.p. 212-214°C.

Example 9

a) A solution of indan-1-one (5.0 g) and *tert*-butyl carbazate (5.0 g) in methanol (20 ml) containing glacial acetic acid (1 drop) was boiled and stirred under reflux for
5 1.5 hours. The mixture was cooled and evaporated under reduced pressure. The residue was recrystallized from ethanol to give indan-1-one *tert*-butyloxycarbonyl-hydrazone.

b) In a similar manner to the previous example, the product from a) (500 mg)
10 was reacted with lithium diisopropylamide (2.23 ml of a 2.0M solution in heptane/THF/ ethylbenzene) and then with ethyl 2-furoate (299 mg) and the compound obtained was treated with trifluoroacetic acid (5 ml) to give 3-(2-furyl)-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 195-197°C.

15 Example 10

In a similar manner to the two previous examples, 6-fluoro-3-(2-thienyl)-1,4-dihydroindeno[1,2-c]pyrazole, m.p. 222-224°C, was prepared starting from 5-fluoroindan-1-one and using ethyl thiophene-2-carboxylate.

20 Examples 11-28 (General Method)

a) A 1:1 mixture (by volume) of dichloromethane:methanol (1 ml) was added via a Gilson 215 liquid handler, to the starting enone (approx. 50 mg, see table I) in a septum sealed tube, followed by aqueous 2M sodium hydroxide solution (1 molar equivalent, see table 1) followed by 100 volumes aqueous hydrogen peroxide (1.8
25 molar equivalents). The resulting solutions/suspensions were then shaken at ambient temperature for 20 hours. Following LCMS analysis, further 100 volumes aqueous hydrogen peroxide (1.8 molar equivalents) was added to each reaction tube, this time manually via a plastic-tipped pipette. Further 1:1 (by volume) dichloromethane: methanol (1 ml) was added to those tubes containing a significant
30 quantity of insoluble material. Shaking was then continued for a further 24 hours.

All reactions were analyzed by t.l.c. and to those judged to have given incomplete reaction was added further 100 volumes aqueous hydrogen peroxide (1.8 molar equivalents) again via a plastic-tipped pipette (see those reactions indicated by x3 in Table I). These reaction mixtures were stirred at ambient temperature for 3 days.

TABLE I

Example	Starting enone (mg)	2N aq. NaOH (μ l)	100 volumes aq. H ₂ O ₂ (μ l)
11	2-(2-Pyridylmethylene)-1-tetralone (51.2 mg)	108.8	44.5 x 2
12	2-(3-Pyridylmethylene)-1-tetralone (49.3 mg)	104.8	42.9 x 2
13	2-(4-Fluorobenzylidene)-1-indanone (50.1 mg)	105.1	43.0 x 2
14	2-Benzylidene-1-benzosuberone (50.3 mg)	101.3	41.4 x 2
15	2-(2-Thienylmethylene)-1-benzosuberone (51.9 mg)	102.0	41.7 x 2
16	2-(4-Methoxybenzylidene)-1-tetralone (50.2 mg)	95.0	38.8 x 2
17	2-(2-Chlorobenzylidene)-1-tetralone (50.4 mg)	93.8	38.4 x 2
18	2-(3-Chlorobenzylidene)-1-tetralone (49.6 mg)	92.3	37.8 x 2
19	2-(4-Chlorobenzylidene)-1-tetralone (51.7 mg)	96.2	39.4 x 2
20	2-(4-Isopropylbenzylidene)-1-tetralone (50.4 mg)	91.2	37.3 x 3
21	2-Piperonylidene-1-tetralone (50.5 mg)	90.7	37.1 x 3
22	6-Methoxy-2-(4-methylbenzylidene)-1-tetralone (51.8 mg)	93.0	38.1 x 3
23	2-(4-Trifluoromethylbenzylidene)-1-indanone (49.3 mg)	85.5	35.0 x 2

TABLE I

Example	Starting enone (mg)	2N aq. NaOH (μ l)	100 volumes aq. H ₂ O ₂ (μ l)
24	2-(2,4-Dichlorobenzylidene)-1-indanone (51.9 mg)	89.7	36.7 x 2
25	2-(2,3-Dimethoxybenzylidene)-1-tetralone (49.6 mg)	84.3	34.5 x 3
26	6-Methoxy-2-(4-methoxybenzylidene)-1-tetralone (50.6 mg)	86.0	35.2 x 3
27	2-(3,5-Dimethoxybenzylidene)-1-tetralone (48.8 mg)	82.9	33.9 x 3
28	5,6-Dimethoxy-2-(4-methoxybenzylidene)-1-indanone (51.5 mg)	83.0	33.9 x 3

The reaction solutions/suspensions were equilibrated between dichloromethane (approx. 5 ml) and water (approx. 2 ml), filtering off the organic phase through an Empore filter cartridge and washing through with dichloromethane (approx. 2 ml). The dichloromethane phases were evaporated and the residues further dried in vacuo.

The products of a) were taken to b) on the basis of being the corresponding 2,3-epoxyketones.

- b) To the products from step 1 in a septum sealed tube was added, via a Gilson 215 liquid handler, n-butanol (2 ml) followed by hydrazine hydrate (2 molar equivalents based on amount of starting enone used in step 1) as a 10% solution by volume in n-butanol (see Table II). To each tube was finally added glacial acetic acid (2 drops) manually by syringe. The reactions were heated at 100° C with shaking for the times indicated in Table II, based on monitoring by t.l.c. analysis. Reaction mixtures were evaporated to dryness and the residues were purified by chromatography on silica eluting with ethyl acetate, diluted where necessary with petroleum ether, b.p. 40-60°C. Appropriate fractions were evaporated and dried in

vacuo to give the final pyrazoles. The identity of the products was confirmed by LCMS using the conditions given below.

LC

- 5 Column: 5 μ HYPERSIL BDS C18 (100 X 2.1 mm)
 Mobile Phase: 0.1% Formic Acid : MeCN (gradient – see below)
 Conditions: 10-100% MeCN in 8 minutes
 (gradient) 100% MeCN for 1 minute
 100-10% MeCN in 2 minutes
 10 (Total analysis run time 11 minutes)
 Flow Rate: 1 ml/min
 Wavelength Range: 206-320 nm
 Injection Volume: 10 μ l

MS

- 15 Ionization APcI +ve/-ve
 Mass Range: 150-500 m/z
 Cone Voltage: 20

The reaction details and yields for the compounds of Examples 11-28 are
 20 shown in Table II:

Table II

Reaction Details

Example	Volume of 10% NH ₂ NH ₂ .H ₂ O μ l	Heating time at 100°C
11	212.0	38 h
12	204.1	38 h
13	204.8	6 h
14	197.3	38 h
15	198.7	22 h
16	185.0	38 h
17	182.7	38 h

Example	Volume of 10% NH ₂ NH ₂ .H ₂ O μ l	Heating time at 100°C
18	179.8	38 h
19	187.4	38 h
20	177.6	22 h
21	176.7	38 h
22	181.3	38 h
23	166.6	6 h
24	174.8	38 h
25	164.1	38 h
26	167.4	22 h
27	161.5	38 h
28	161.6	6 h

Table II (continued)

Yields/LCMS

Example	MF	MW	MH ⁺ found	Retention time (mins)	Final Mass
11	C ₁₆ H ₁₃ N ₃	247	Yes	4.26	15.9 mg
12	C ₁₆ H ₁₃ N ₃	247	Yes	3.74	10.4 mg
13	C ₁₆ H ₁₁ FN ₂	250	Yes	4.60	24.9 mg
14	C ₁₈ H ₁₆ N ₂	260	Yes	5.10	15.8 mg
15	C ₁₆ H ₁₄ N ₂ S	266	Yes	5.07	24.1 mg
16	C ₁₈ H ₁₆ N ₂ O	276	Yes	4.75	23.6 mg
17	C ₁₇ H ₁₃ ClN ₂	280.5	Yes	5.03	4.2 mg
18	C ₁₇ H ₁₃ ClN ₂	280.5	Yes	5.38	16.7 mg
19	C ₁₇ H ₁₃ ClN ₂	280.5	Yes	5.40	15.7 mg
20	C ₂₀ H ₂₀ N ₂	288	Yes	5.87	15.0 mg
21	C ₁₈ H ₁₄ N ₂ O ₂	290	Yes	4.70	11.9 mg
22	C ₁₉ H ₁₈ N ₂ O	290	Yes	5.06	7.6 mg

Example	MF	MW	MH ⁺ found	Retention time (mins)	Final Mass
23	C ₁₇ H ₁₁ F ₃ N ₂	300	Yes	5.32	3.9 mg
24	C ₁₆ H ₁₀ Cl ₂ N ₂	301	Yes	5.45	5.1 mg
25	C ₁₉ H ₁₈ N ₂ O ₂	306	Yes	4.77	10.4 mg
26	C ₁₉ H ₁₈ N ₂ O ₂	306	Yes	4.64	9.8 mg
27	C ₁₉ H ₁₈ N ₂ O ₂	306	Yes	4.91	11.7 mg
28	C ₁₉ H ₁₈ N ₂ O ₃	322	Yes	3.93	29.1 mg

The compounds produced by Examples 11-28 are:

Example 11

- 5 3-(2-Pyridyl)-4,5-dihydro-2*H*-benz[g]indazole

Example 12

3-(3-Pyridyl)-4,5-dihydro-2*H*-benz[g]indazole

10 Example 13

3-(4-Fluorophenyl)-1,4-dihydroindeno[1,2-*c*]pyrazole

Example 14

3-Phenyl-2,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole

15

Example 15

3-(2-Thienyl)-2,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole

Example 16

- 20 3-(4-Methoxyphenyl)-4,5-dihydro-2*H*-benz[g]indazole

Example 17

3-(2-Chlorophenyl)-4,5-dihydro-2*H*-benz[g]indazole

Example 18

5 3-(3-Chlorophenyl)-4,5-dihydro-2*H*-benz[g]indazole

Example 19

3-(4-Chlorophenyl)-4,5-dihydro-2*H*-benz[g]indazole

10 Example 20

3-(4-Isopropylphenyl)-4,5-dihydro-2*H*-benz[g]indazole

Example 21

3-Piperonyl-4,5-dihydro-2*H*-benz[g]indazole

15

Example 22

7-Methoxy-3-(4-tolyl)-4,5-dihydro-2*H*-benz[g]indazole

Example 23

20 3-(4-Trifluoromethylphenyl)-1,4-dihydroindeno[1,2-*c*]pyrazole

Example 24

3-(2,4-Dichlorophenyl)-1,4-dihydroindeno[1,2-*c*]pyrazole

25 Example 25

3-(2,3-Dimethoxyphenyl)-4,5-dihydro-2*H*-benz[g]indazole

Example 26

7-Methoxy-3-(4-methoxyphenyl)-4,5-dihydro-2*H*-benz[g]indazole

30

Example 27

3-(3,5-Dimethoxyphenyl)-4,5-dihydro-2H-benz[g]indazole

Example 28

5 6,7-Dimethoxy-3-(4-methoxyphenyl)-1,4-dihydroindeno[1,2-c]pyrazole

Example 29

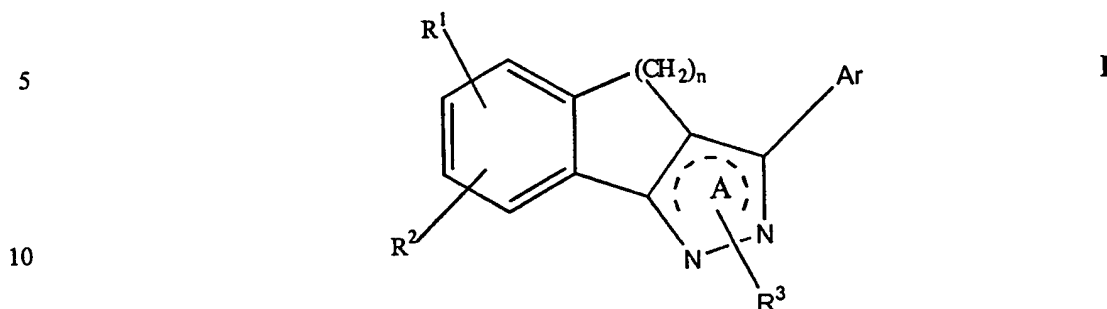
a) Indan-1-one (3.30 g), thiophene-3-carboxaldehyde (2.60 ml), acetic acid
10 (0.47 ml) and piperidine (0.57 ml) were heated together on a steam-bath for
16 hours. The mixture was cooled to give a solid mass which was boiled with IMS
(120 ml) and hot filtered to remove insoluble material. The filtrate was cooled in ice
to precipitate a brown solid which was collected by filtration and dried to give 2-(3-
thienyl)methyleneindan-1-one, m.p. 137-138°C.

15

b) A mixture of the product from a) (2.03 g), p-toluenesulfonyl hydrazine
(2.00 g), p-toluenesulfonic acid (0.35 g) and ethanol (30 ml) was boiled under reflux
for 44 hours. The solvent was removed under reduced pressure and the residue was
dissolved in dichloromethane (100 ml). The solution was washed with 2M aqueous
20 sodium hydroxide solution (25 ml). The organic phase was dried, filtered through a
silica pad, washing with ethyl acetate, and evaporated to give an oil which was
purified by flash column chromatography on silica using petroleum ether, b.p.
60-80°C/ethyl acetate (2:1) followed by the same solvents in the ratio of 1:1 as the
mobile phase. Appropriate fractions were combined and evaporated to give a solid
25 which was triturated with diethyl ether and filtered to give 3-(3-thienyl)-1,4-
dihydroindeno[1,2-c]pyrazole, m.p. 219-220°C.

The chemical structures of the compounds produced in these Examples are
shown in Table III:

TABLE III



Ex. No.	n	Ar	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
1	1	3-pyridyl	H	H	H	H	H	H
2	1	4-pyridyl	H	H	H	H	H	H
3	2	2-thienyl	H	H	H	H	H	H
4	1	Phenyl	H	H	H	H	H	H
5	1	phenyl (sub)	H	H	H	3-OCH ₃	H	H
6	1	phenyl (sub)	H	H	H	4-OCH ₃	H	H
7	1	phenyl (sub)	H	H	H	4-CH ₃	H	H
8	1	2-thienyl	6-OCH ₃	H	H	H	H	H
9	1	2-furyl	H	H	H	H	H	H
10	1	2-thienyl	6-F	H	H	H	H	H
11	2	2-pyridyl	H	H	H	H	H	H
12	2	3-pyridyl	H	H	H	H	H	H
13	1	phenyl (sub)	H	H	H	4-F	H	H
14	3	Phenyl	H	H	H	H	H	H
15	3	2-thienyl	H	H	H	H	H	H
16	2	phenyl (sub)	H	H	H	4-OCH ₃	H	H
17	2	phenyl (sub)	H	H	H	2-Cl	H	H
18	2	phenyl (sub)	H	H	H	3-Cl	H	H
19	2	phenyl (sub)	H	H	H	4-Cl	H	H
20	2	phenyl (sub)	H	H	H	4-isoPr	H	H

Ex. N .	n	Ar	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
21	2	phenyl (sub)	H	H	H	3,4-OCH ₂ O-		H
22	2	phenyl (sub)	7-OCH ₃	H	H	4-CH ₃	H	H
23	2	phenyl (sub)	H	H	H	4-CF ₃	H	H
24	1	phenyl (sub)	H	H	H	2-Cl	4-Cl	H
25	2	phenyl (sub)	H	H	H	2-OCH ₃	3-OCH ₃	H
26	2	phenyl (sub)	7-OCH ₃	H	H	4-OCH ₃	H	H
27	2	phenyl (sub)	H	H	H	3-OCH ₃	5-OCH ₃	H
28	1	phenyl (sub)	6-OCH ₃	7-OCH ₃	H	4-OCH ₃	H	H
29	1	3-thienyl	H	H	H	H	H	H

II. *In Vitro* PTK Assays

The following *in vitro* assays may be used to determine the level of activity and effect of the different compounds of the present invention on one or more of the PTKs. Similar assays can be designed along the same lines for other tyrosine kinases using techniques well known in the art.

A. KDR Protein Production Using Baculovirus System:

The coding sequence for the KDR intra-cellular domain (aa789-1354) was generated through PCR using cDNAs isolated from HUVEC cells. A poly-His₆ sequence was introduced at the N-terminus of this protein as well. This fragment was cloned into transfection vector pVL1393 at the Xba 1 and Not 1 site. Recombinant baculovirus (BV) was generated through co-transfection using the BaculoGold Transfection reagent (PharMingen). Recombinant BV was plaque purified and verified through Western analysis. For protein production, SF-9 cells were grown in SF-900-II medium at 2×10^6 /ml, and were infected at 0.5 plaque forming units per cell (MOI). Cells were harvested at 48 hours post infection.

B. Purification of KDR

SF-9 cells expressing (His)₆KDR(aa789-1354) were lysed by adding 50 ml of Triton X-100 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1mM PMSF, 10µg/ml aprotinin, 1 µg/ml leupeptin) to the cell pellet
5 from 1L of cell culture. The lysate was centrifuged at 19,000 rpm in a Sorval SS-34 rotor for 30 min at 4°C. The cell lysate was applied to a 5 ml NiCl₂ chelating sepharose column, equilibrated with 50 mM HEPES, pH7.5, 0.3 M NaCl. KDR was eluted using the same buffer containing 0.25 M imidazole. Column fractions were analyzed using SDS-PAGE and an ELISA assay (below) which measures kinase
10 activity. The purified KDR was exchanged into 25mM HEPES, pH7.5, 25mM NaCl, 5 mM DTT buffer and stored at -80°C.

C. Human Tie-2 Kinase Production and Purification

The coding sequence for the human Tie-2 intra-cellular domain (aa775-1124)
15 was generated through PCR using cDNAs isolated from human placenta as a template. A poly-His₆ sequence was introduced at the N-terminus and this construct was cloned into transfection vector pVL 1939 at the Xba 1 and Not 1 site. Recombinant BV was generated through co-transfection using the BaculoGold Transfection reagent (PharMingen). Recombinant BV was plaque purified and
20 verified through Western analysis. For protein production, SF-9 insect cells were grown in SF-900-II medium at 2 x 10⁶/ml, and were infected at MOI of 0.5. Purification of the His-tagged kinase used in screening was analogous to that described for KDR.

25 D. Human Flt-1 Tyrosine Kinase Production and Purification

The baculoviral expression vector pVL1393 (Phar Mingen, Los Angeles, CA) was used. The nucleotide sequence encoding the kinase domain was generated through PCR using cDNA libraries isolated from HUVEC cells. A nucleotide sequence encoding poly-His₆ was placed 5' to the nucleotide region encoding the
30 entire intracellular kinase domain of human Flt-1 (amino acids 786-1338). The histidine residues enabled affinity purification of the protein as a manner analogous

to that for KDR (Part B.) and ZAP70 (Part F.) SF-9 insect cells were infected at a 0.5 multiplicity and harvested 48 hours post infection.

E. Lck and EGFR Tyrosine Kinase Sources

5 Lck or truncated forms of Lck were commercially obtained (e.g. Upstate Biotechnology Inc., Saranac Lake, NY or Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or were purified from known natural or recombinant sources using conventional methods. EGFR was purchased from Sigma (Cat # E-3641; 500 units/50 µl) and the EGF ligand was acquired from Oncogene Research
10 Products/Calbiochem (Cat # PF011-100).

F. ZAP70 Tyrosine Kinase Production

The Baculoviral expression vector pVL 1393 (Phar Mingen, Los Angeles, CA) was used. A nucleotide sequence encoding poly-His₆ was placed 5' to the
15 nucleotide region encoding the entire ZAP70 (amino acids 1-619). The nucleotide sequence encoding the ZAP70 coding region was generated through PCR using cDNA libraries isolated from Jurkat immortalized T-cells. The histidine residues enabled affinity purification of the protein. The LVPRGS bridge constituted a recognition sequence for proteolytic cleavage by thrombin, thereby enabling
20 removal of the affinity tag from the enzyme. SF-9 insect cells were infected at a 0.5 multiplicity and harvested 48 hours post infection.

G. Purification of ZAP70

SF-9 cells were lysed in a buffer containing 20 mM Tris, pH 8.0, 137 mM
25 NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM sodium orthovanadate. The soluble lysate was applied to a chelating Sepharose Hi Trap column (Pharmacia) equilibrated in 50 mM HEPES, pH 7.5, 0.3 M NaCl. The fusion protein was eluted with 250 mM imidazole. The recovered enzyme was stored in buffer containing 50 mM HEPES, pH 7.5, 50 mM
30 NaCl and 5 mM DTT.

H. Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme linked immunosorbent assays (ELISA) can be used to detect and measure the presence of tyrosine kinase activity. The ELISA can be conducted according to known protocols which are described in, for example, Voller, *et al.*,
5 1980, "Enzyme-Linked Immunosorbent Assay," In: *Manual of Clinical Immunology*,
2d ed., edited by Rose and Friedman, pp 359-371 Am. Soc. of Microbiology,
Washington, D.C.

The disclosed protocol can be adapted for determining activity with respect to a specific RTK. For example, preferred protocols for conducting the ELISA
10 experiments for KDR is provided below. Adaptation of these protocols for
determining a compound's activity for other members of the RTK family, as well as
non-receptor tyrosine kinases, are well within the abilities of those in the art. For
purposes of determining inhibitor selectivity, a universal PTK substrate (e.g.,
random copolymer of poly (Glu₄ Tyr) 20,000-50,000 MW) was employed together
15 with ATP (typically 5 μ M) at concentrations approximately twice the apparent K_m in
the assay.

KDR *IN VITRO* ELISA

The following procedure was used to assay the inhibitory effect of
20 compounds of this invention on KDR tyrosine kinase activity:

Buffers and Solutions:

1. PGT: Poly (Glu,Tyr) 4:1

Store powder at -20°C. Dissolve powder in phosphate buffered saline (PBS)
for 50mg/ml solution. Store 1ml aliquots at -20°C. When making plates dilute to
25 250 μ g/ml in Gibco PBS.

2. Reaction Buffer:

100mM Hepes, 20mM MgCl₂, 4mM MnCl₂, 5mM DTT, 0.02%BSA,
200 μ M NaVO₄, pH 7.10

30

3. ATP: Store aliquots of 100mM at -20°C. Dilute to 20 μ M in water

4. Washing Buffer:
 PBS with 0.1% Tween 20
- 5 5. Antibody Diluting Buffer:
 0.1% bovine serum albumin (BSA) in PBS
6. TMB Substrate:
 Mix TMB substrate and Peroxide solutions 9:1 just before use or use K-Blue
- 10 Substrate from Neogen
7. Stop Solution:
 1M Phosphoric Acid
- 15 Procedure
1. Plate Preparation:
 Dilute PGT stock (50mg/ml, frozen) in PBS to a 250µg/ml. Add 125µl per
 well of Corning modified flat bottom high affinity ELISA plates (Corning #25805-
 96). Add 125µl PBS to blank wells. Cover with sealing tape and incubate overnight
20 37°C. Wash 1x with 250µl washing buffer and dry for about 2 hrs in 37°C dry
 incubator.
 Store coated plates in sealed bag at 4°C until used.
2. Tyrosine Kinase Reaction:
25 -Prepare inhibitor solutions at a 4x concentration in 20% DMSO in water.
 -Prepare reaction buffer
 -Prepare enzyme solution so that desired units are in 50µl, e.g. for KDR
 make to 1 ng/µl for a total of 50ng per well in the reactions. Store on ice.
 -Make 4x ATP solution to 20µM from 100mM stock in water. Store on ice.
30 -Add 50µl of the enzyme solution per well.
 -Add 25µl 4x inhibitor.

-41-

- Add 25 μ l 4x ATP for inhibitor assay.
- Incubate for 10 minutes at room temperature.
- Stop reaction by adding 50 μ l 0.05N HCl per well.
- Wash plate.

5

****Final Concentrations for Reaction:**ATP: 5 μ M

5% DMSO

10 3. Antibody Binding

- Dilute 1mg/ml aliquot of PY20-HRP (Pierce) antibody to 50ng/ml in 0.1% BSA in PBS by a 2 step dilution (100x, then 200x).
- Add 100 μ l Ab per well. Incubate 1 hr at room temp. Incubate 1 hr at 4C.
- Wash 4x plate.

15

4. Color reaction

- Prepare TMB substrate and add 100 μ l per well.
- Monitor OD at 650nm until 0.6 is reached.
- Stop with 1M Phosphoric acid. Shake on plate reader.

20

Optimal incubation times and enzyme reaction conditions vary slightly with enzyme preparations and are determined empirically for each lot.

Analogous assay conditions were used for Flt-1, Tie-2, EGFR and ZAP70. For Lck, the Reaction Buffer utilized was 100 mM MOPSO, pH 6.5, 4 mM MnCl₂, 20 mM MgCl₂, 5 mM DTT, 0.2% BSA, 200 mM NaVO₄ under the analogous assay conditions.

25

PKC kinase source

The catalytic subunit of PKC may be obtained commercially (Calbiochem).

30

PKC kinase assay

A radioactive kinase assay was employed following a published procedure (Yasuda, I., Kirshimoto, A., Tanaka, S., Tominaga, M., Sakurai, A., Nishizuka, Y. *Biochemical and Biophysical Research Communication* 3:166, 1220-1227 (1990)).

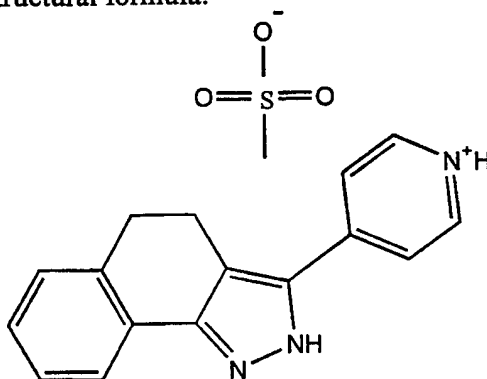
- 5 Briefly, all reactions were performed in a kinase buffer consisting of 50 mM Tris-HCl pH7.5, 10mM MgCl₂, 2mM DTT, 1mM EGTA, 100 μ M ATP, 8 μ M peptide, 5% DMSO and ³³P ATP (8Ci/mM). Compound and enzyme were mixed in the reaction vessel and the reaction initiated by addition of the ATP and substrate mixture. Following termination of the reaction by the addition of 10 μ L stop buffer
- 10 (5 mM ATP in 75mM phosphoric acid), a portion of the mixture was spotted on phosphocellulose filters. The spotted samples were washed 3 times in 75 mM phosphoric acid at room temperature for 5 to 15 minutes. Incorporation of radiolabel was quantified by liquid scintillation counting.

15 Estrogen Receptor Binding Assay

- Binding of 1 nM radiolabeled 17 β -estradiol to human estrogen receptor in the cytosol of MCF-7 mammary carcinoma cells was determined following incubation for 20 h at 4° C using the reaction conditions of Shein *et al.*, *Cancer Res.* 45:4192 (1985) (herein incorporated by reference). Following incubation, the
- 20 cytosol fractions were mixed with a suspension of dextran-coated charcoal for 10 min at 4°C, centrifuged, and the supernatants collected. Bound radioactivity remaining in the charcoal supernatant was measured with a scintillation counter (LS 6000, Beckman) using a liquid scintillation cocktail (Formula 989 Packard). The compounds were tested simultaneously at eight concentrations in duplicate to obtain
- 25 a competition curve in order to quantify the inhibitory activity. The specific radioligand binding to the estrogen receptor was defined as the difference between total binding and nonspecific binding determined in the presence of an excess of unlabeled 17 -estradiol (6 μ M).

Results

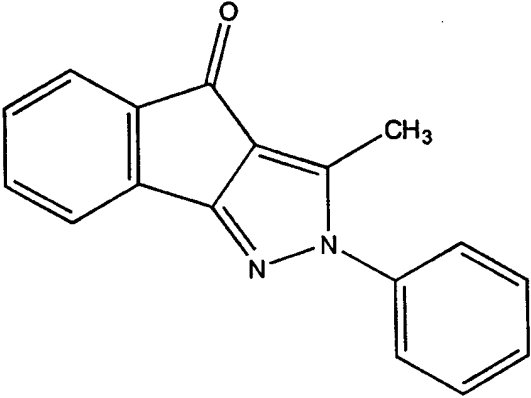
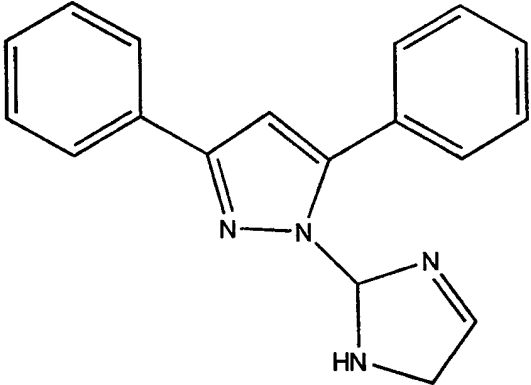
The following inhibitory concentrations of a representative compound (Example 4) with the structural formula: -



were obtained:

Assay	IC ₅₀ for Compound
KDR	0.20 μM
Flt-1	> 50.0 μM
Lck	> 50.0 μM
TIE2	> 50.0 μM
ZAP70	> 50.0 μM
EGFR	> 50.0 μM
PKC	≥ 20 μM
Estrogen Receptor	>10.0 μM (< 10% inh @ 10 μM)

In addition, the following inhibitory concentration of another representative compound was obtained:

	Compound of Formula I where:	KDR ELISA IC ₅₀
5	R ¹ is hydrogen, R ² is hydrogen, R ³ is hydrogen, n=1 and Ar is 2-thienyl	0.15 μM
10		>100 μM
15		>100 μM
20		

These results demonstrate that compounds of the present invention and exemplified herein have notable inhibitory activity for KDR tyrosine kinase and are particularly selective as KDR tyrosine kinase inhibitors.

III. Cellular RTK Assays

The following cellular assays may be used to determine the level of activity and effect of the different compounds of the present invention on one or more of the RTKs. Similar assays can be designed along the same lines for other tyrosine kinases using techniques well known in the art.

A. VEGF-Induced KDR Phosphorylation in Human Umbilical Vein Cells (HUVEC) as Measured by Western Blots.

1. HUVEC cells (from pooled donors) were purchased from Clonetics (San Diego, CA) and cultured according to the manufacturer directions. Only early passages (3-8) were used for this assay. Cells were cultured in 100 mm dishes (Falcon for tissue culture; Becton Dickinson; Plymouth, England) using complete EBM media (Clonetics).
2. For evaluating a compound's inhibitory activity, cells were trypsinized and seeded at $0.5-1.0 \times 10^5$ cells/well in each well of 6-well cluster plates (Costar; Cambridge, MA).
3. 3-4 days after seeding, plates were 90-100% confluent. Medium was removed from all the wells, cells were rinsed with 5-10ml of PBS and incubated 18-24h with 5ml of EBM base media with no supplements added (i.e., serum starvation).
4. Serial dilutions of inhibitors were added in 1ml of EBM media (25uM, 5uM, or 1uM final concentration) to cells and incubated for one hour at 37 C. Human recombinant VEGF₍₁₆₅₎ (R&D Systems) was then added to all the wells in 2 ml of EBM medium at a final concentration of 50ng/ml and incubated at 37 C for 10 minutes. Control cells untreated or treated with VEGF only were used to assess background phosphorylation and induction by VEGF.
5. All wells were then rinsed with 5-10ml of cold PBS containing 1mM Sodium Orthovanadate (Sigma) and cells were lysed and scraped in 200µl of RIPA buffer (50mM Tris-HCl pH7, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1mM EDTA) containing protease inhibitors (PMSF 1mM, Aprotinin 1µg/ml, Pepstatin 1µg/ml, Leupeptin 1µg/ml, Na Vanadate 1mM,

Na Fluoride 1mM) and 1 μ g/ml of DNase (all chemicals from Sigma Chemical Company, St. Louis, MO). The lysate was spun at 14,000 rpm for 30min, to eliminate nuclei.

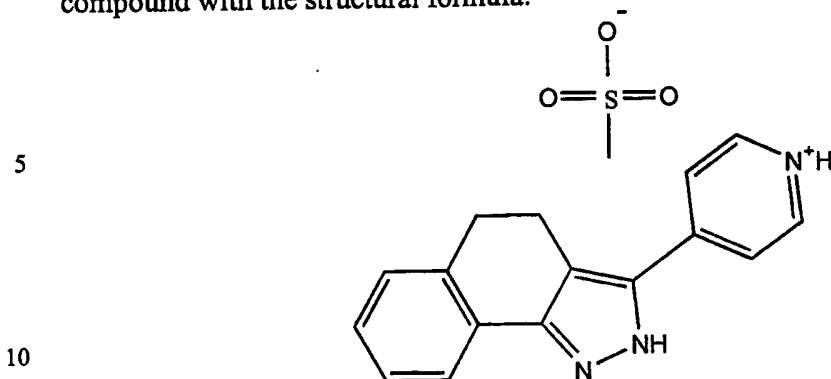
5 6. Equal amounts of proteins were then precipitated by addition of cold
(-20 C) Ethanol (2 volumes) for a minimum of 1 hour or a maximum of
overnight. Pellets were reconstituted in Laemli sample buffer containing 5%
 β -mercaptoethanol (BioRad; Hercules, CA) and boiled for 5min. The
proteins were resolved by polyacrylamide gel electrophoresis (6%, 1.5mm
10 Novex, San Deigo, CA) and transferred onto a nitrocellulose membrane
using the Novex system. After blocking with bovine serum albumin (3%),
the proteins were probed overnight with anti-KDR polyclonal antibody (C20,
Santa Cruz Biotechnology,, Santa Cruz, CA) or with anti-phosphotyrosine
monoclonal antibody (4G10, Upstate Biotechnology, Lake Placid, NY) at
15 4°C. After washing and incubating for 1 hour with HRP-conjugated F(ab)₂ of
goat anti-rabbit or goat-anti-mouse IgG the bands were visualized using the
emission chemiluminescence (ECL) system (Amersham Life Sciences,
Arlington Height, IL).

20 Results

The following inhibitory concentration of a representative compound was obtained:

Compound of Formula I where:	Cellular VEGF-Induced KDR Phosphorylation IC ₅₀
R ¹ is hydrogen, R ² is hydrogen, R ³ is hydrogen, n=1 and Ar is 2-thienyl	3.0 μ M

In addition, the following inhibitory concentrations of another representative compound with the structural formula:



were:

15

HUVEC	Cellular IC ₅₀ for Compound
KDR phosphorylation	5-10 μ M

This compound also has demonstrated tyrosine kinase selectivity.

These results demonstrate that compounds of the present invention have notable inhibitory activity for VEGF-induced tyrosine phosphorylation of KDR tyrosine kinase in endothelial cells.

B. HUVEC Mitogenesis Assay

Human umbilical vein endothelial cells (HUVEC) were cultured in EGM-Complete Media (endothelial growth media supplemented with 10% FBS, hydrocortisone, epidermal growth factor and bovine brain extract as per the supplier's directions). Cells, media and supplements were purchased from Clonetics (San Diego, CA). The HUVEC Mitogenesis Assay was used to measure inhibition of mitogenesis induced by VEGF, FGF or Complete Media. Cells were harvested by trypsinization, washed once and suspended in Complete Media (CM) at a concentration of 5,000 cells/0.15 ml. Cells were seeded into 96-well plates at 5,000 cells/well and incubated at 37 C for 24 hours. Cells were washed once with serum-free media (SFM) and incubated under serum-free conditions for 24 hours. Non-serum starved control wells were cultured with CM during this period. Test

compounds were prepared as 10 mM stock solutions in DMSO, diluted in SFM and added to wells over a concentration range (0.005-50 μ M final concentration). Cells were incubated for 1 hour prior to addition of 50 ng/ml VEGF or FGF (R & D Systems, Minneapolis, MN). Complete media stimulated wells received the test compound and stimulus simultaneously. After 5 hours of incubation, 0.5 Ci/well of tritiated thymidine (Amersham) was added and the cells were incubated overnight. The assay was stopped by freezing plates at -20 C for 24 hours. Plates were harvested onto filter mats with a Tomtec Harvester and counted with a Betaplate liquid scintillation counter (Wallac).

10

Results

The following inhibitory concentration of a representative compound was obtained:

Compound of Formula I where:	VEGF-Stimulated Mitogenesis IC ₅₀
R ¹ is hydrogen, R ² is hydrogen, R ³ is hydrogen, n=1 and Ar is 2-thienyl	2.0 μ M

15

These results demonstrate that compounds of the present invention have notable inhibitory activity for VEGF-stimulated mitogenesis.

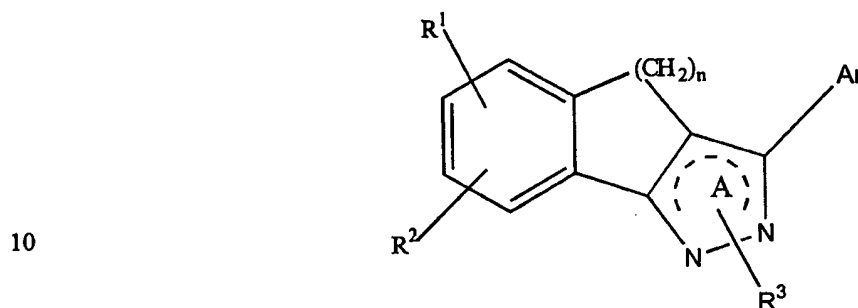
While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

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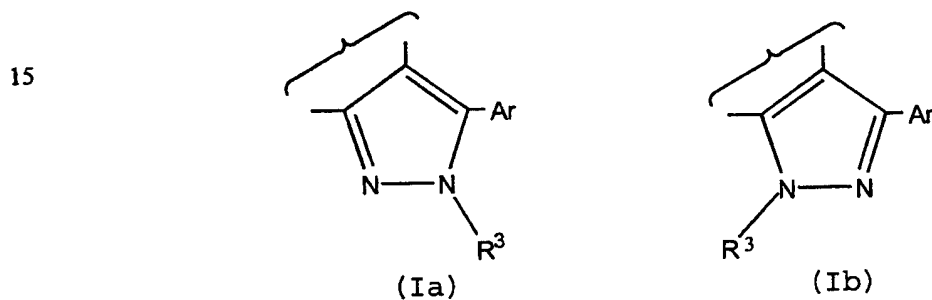
CLAIMS

What is claimed is:

1. A method of inhibiting tyrosine kinase activity comprising the administration
 5 of a compound represented by the formula:



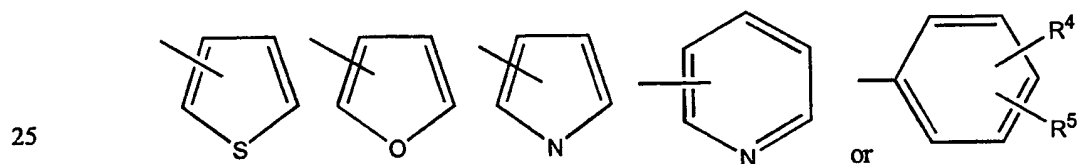
where ring A represents the tautomeric structures



20

n is 1, 2, or 3;

Ar is

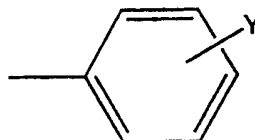


and

30 R^1 , R^2 , R^4 and R^5 each independently represent hydrogen, halogen having an atomic weight of about 19 to 36, lower alkyl having 1 to 4 carbon atoms, lower alkoxy, trifluoromethyl or

R^1 , R^2 and R^4 , R^5 together independently represent methylenedioxy attached to adjacent carbon atoms;

- 5 R^3 is hydrogen, $-\text{COR}^6$, or $-\text{SO}_2\text{R}^6$; and
 R^6 is $-\text{CH}_3$ or



10

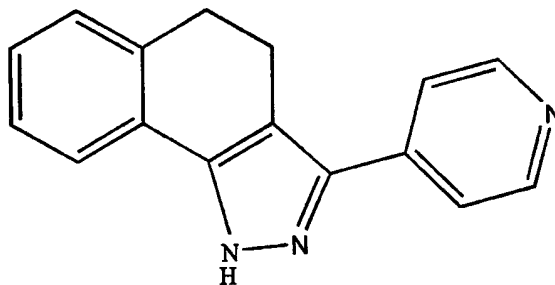
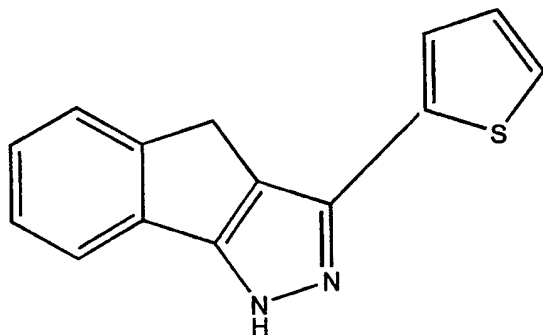
wherein Y is hydrogen, chlorine or $-\text{CH}_3$

to said tyrosine kinase in sufficient concentration to inhibit the enzyme activity of said tyrosine kinase.

15

2. The method of Claim 1 wherein said compound is in an enantiomeric form, is in a mixture with one or more other said compounds, or is both in an enantiomeric form and in a mixture with one or more other said compounds.
- 20 3. The method of Claim 1 wherein said tyrosine kinase is either a receptor tyrosine kinase or a non-receptor tyrosine kinase.
4. The method of Claim 3 wherein said tyrosine kinase is selected from the group consisting of KDR, flt-1, TIE-2, Lck, Src, fyn and yes.
- 25 5. The method of Claim 1 wherein the activity of said tyrosine kinase affects angiogenesis.
6. The method of Claim 5 wherein the inhibition of said tyrosine kinase is anti-angiogenic.
- 30

7. The method of Claim 6 wherein said inhibition of said tyrosine kinase inhibits the progression of a disease state selected from the group consisting of hyperproliferative disorders, arthritis, atherosclerosis, psoriasis, hemangioma, myocardial angiogenesis, coronary and cerebral collaterals, ischemic limb angiogenesis, corneal disease, rubeosis, neovascular glaucoma, macular degeneration, wound healing, peptic ulcer *Helicobacter* related diseases, fractures, diabetic retinopathy, and cat scratch fever.
8. The method of Claim 1 wherein the activity of said tyrosine kinase affects vascular hyperpermeability.
9. The method of Claim 1 wherein n is 1; R³ is hydrogen; R¹ is selected from the group consisting of hydrogen, methoxy and fluoro; and Ar is selected from the group consisting of 2-thienyl, 3-thienyl and 2-furanyl.
10. The method of Claim 1 wherein said compound is



or

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21258

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K31/415 A61K31/44 C07D401/04 C07D409/04 C07D231/56
C07D405/04 C07D231/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EL-RAYYES N R ET AL: "Heterocycles. Part XVII. Synthesis of new substituted 2,3,3a,4,5,6-hexahydrobenzo[6,7]cyclohepta[1,2-c]pyrazoles and related compounds" J. HETEROCYCL. CHEM. (JHTCAD,0022152X);89; VOL.26 (1); PP.209-14, XP002094611 Kuwait Univ.;Dep. Chem.; Kuwait; Kuwait (KW) see page 209; figure 1 --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

24 February 1999

Date of mailing of the international search report

10/03/1999

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Authorized officer

Paisdor, B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21258

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YOSHIMURA H ET AL: "A Novel Type of Retinoic Acid Receptor Antagonist: Synthesis and Structure-Activity Relationships of Heterocyclic Ring-Containing Benzoic Acid Derivatives" J. MED. CHEM. (JMCMAR,00222623);95; VOL.38 (16); PP.3163-73, XP002094612 Eisai Company Ltd.;Tsukuba Research Laboratories; Tsukuba; 300-26; Japan (JP) see abstract see page 3163, column 1, paragraph 1 - column 2	1-13
A	--- PALMER B D ET AL: "Tyrosine Kinase Inhibitors. 11. Soluble Analogs of Pyrrolo- and Pyrazoloquinazolines as Epidermal Growth Factor Receptor Inhibitors: Synthesis, Biological Evaluation, and Modeling of the Mode of Binding" J. MED. CHEM. (JMCMAR,00222623);97; VOL.40 (10); PP.1519-1529, XP002094613 University of Auckland School of Medicine;Cancer Society Research Laboratory Faculty of Medicine and Health Science; Auckland; 1000; N. Z. (NZ) see abstract see page 1520, column 2, last paragraph - page 1521, column 1; table 1 see page 1522; table 2	1-13
A	--- US 3 843 665 A (COOMBS R V ET AL) 22 October 1974 see column 1 see column 4 - column 8; examples	1-13
A	--- US 3 932 430 A (HABECK D A ET AL) 13 January 1976 see column 13 - column 23; claim 1; examples 1-12	1-13
A	--- WO 96 14843 A (COR THERAPEUTICS, INC.;USA) 23 May 1996 see abstract; claim 1	1-13
A	--- WO 94 14777 A (EISAI CO., LTD.;JAPAN) 7 July 1994 see abstract see claims 1,4	1-13
A	--- WO 96 31510 A (CIBA-GEIGY A.-G.;SWITZ.) 10 October 1996 see page 6, line 8 - page 7, line 5 see abstract; claims 1,4	1-13
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INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/US 98/21258

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 92 20642 A (RHONE POULENC RORER INT) 26 November 1992 cited in the application see abstract; claim 6 see page 42 - page 44 see page 45 - page 48 -----</p>	1-13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 21258

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-13
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-13
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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